

EXHIBIT N

TO DECLARATION OF SCOTT D. TANNER, PHD.

U.S. Patent Application Ser. No. 10/614,115

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Determination of phosphorus in small amounts of protein samples by ICP–MS

Received: 20 September 2002 / Revised: 29 November 2002 / Accepted: 2 December 2002 / Published online: 11 February 2003
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Abstract Inductively coupled plasma mass spectrometry (ICP–MS) is used for phosphorus determination in protein samples. A small amount of solid protein sample (down to 1 µg) or digest (1–10 µL) protein solution was denatured in nitric acid and hydrogen peroxide by closed-microvessel microwave digestion. Phosphorus determination was performed with an optimized analytical method using a double-focusing sector field inductively coupled plasma mass spectrometer (ICP–SFMS) and quadrupole-based ICP–MS (ICP–QMS). For quality control of phosphorus determination a certified reference material (CRM), single cell proteins (BCR 273) with a high phosphorus content of $26.8 \pm 0.4 \text{ mg g}^{-1}$, was analyzed. For studies on phosphorus determination in proteins while reducing the sample amount as low as possible the homogeneity of CRM BCR 273 was investigated. Relative standard deviation and measurement accuracy in ICP–QMS was within 2%, 3.5%, 11% and 12% when using CRM BCR 273 sample weights of 40 mg, 5 mg, 1 mg and 0.3 mg, respectively. The lowest possible sample weight for an accurate phosphorus analysis in protein samples by ICP–MS is discussed. The analytical method developed was applied for the analysis of homogeneous protein samples in very low amounts [1–100 µg of solid protein sample, e.g. β -casein or down to 1 µL of protein or digest in solution (e.g., tau protein)]. A further reduction of the diluted protein solution volume was achieved by the application of flow injection in ICP–SFMS, which is discussed with reference to real protein digests after protein separation using 2D gel electrophoresis.

The detection limits for phosphorus in biological samples were determined by ICP–SFMS down to the ng g^{-1} level.

The present work discusses the figure of merit for the determination of phosphorus in a small amount of protein sample with ICP–SFMS in comparison to ICP–QMS.

Keywords ICP–SFMS · ICP–QMS · Proteins · Phosphorus

Introduction

Of special importance in life sciences is the determination of phosphorus, especially in protein samples. Phosphorylation is probably one of the most biologically important modifications of proteins, and is implicated both in normal physiology and in human disease, carcinogenesis, and aging [1, 2]. The relation of phosphorylation to many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, etc., is recognized today. Interest in phosphorus determination focuses on the development and application of analytical methods for determining the total phosphorus content [3, 4] in proteins or different species of this element [5, 6].

With its ability to provide very sensitive, accurate and precise multielement determinations of trace elements and of isotope ratios, inductively coupled plasma mass spectrometry (ICP–MS) [7, 8, 9] has developed into one of the most important inorganic mass spectrometric techniques. One serious disadvantage of ICP–MS, which is the limiting factor of this analytical method, is that a multitude of different isobaric interferences with atomic and molecular ions appear. The determination of phosphorus—as a mono-isotope element at mass 31 u—is disturbed especially by interferences of the following molecular ions $^{15}\text{N}^{16}\text{O}^+$, $^{14}\text{N}^{17}\text{O}^+$, $^{14}\text{N}^{16}\text{O}^+\text{H}^+$ and $^{30}\text{Si}^1\text{H}^+$, formed in an argon plasma and by expansion of plasma in the interface behind the sampler cone. For the separation of isobaric interferences of $^{31}\text{P}^+$ with molecular ions a mass resolution ($m/\Delta m$) better than 1500 is required. Furthermore, the phosphorus determination is disturbed by isobaric interferences with the double-charged atomic ion $^{62}\text{Ni}^{2+}$. Therefore, the use of a double-focusing sector field ICP–MS (ICP–SFMS) at the

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required mass resolution is advantageous for phosphorus determination in small sample amounts of any matrix. In addition, phosphorus is a minor element with a mean concentration of $1000 \mu\text{g g}^{-1}$ in the earth's crust (comparable with F or Mn). Due to the relatively high concentration in nature (in particular, in human tissue and fluids) there is a great danger of contamination of protein sample with free phosphates originating both from ambient fluids or from laboratory environment including chemicals, etc. In order to avoid contamination during the sample preparation, closed-vessel microwave digestion of protein samples or direct sampling, e.g. via laser ablation (LA), can be applied. In addition, special separation procedures are often required to distinguish phosphorus in the object of interest from the phosphorus originating from the environment.

Thus, in order to determine the degree of protein phosphorylation Wind et al. [5] coupled a capillary liquid chromatograph to a sector field ICP-MS and applied the method to phosphoproteins, e.g. α - or β -casein. The potential of ICP-MS with a collision cell for phosphorus determination in proteins and the determination of the state of phosphorylation of proteins was discussed by Baranov et al. [4]. Using a quadrupole ICP-MS (ICP-QMS) with dynamic reaction cell the authors analyzed phosphorus via the $^{31}\text{P}^{16}\text{O}^+$ molecular ions and avoided isobaric interferences at mass 31 u. However, new interferences at mass 47 u (e.g. $^{47}\text{Ti}^+$, $^{94}\text{Zr}^{2+}$, $^{15}\text{N}^{16}\text{O}_2^+$ and others) must be considered; that means this analytical procedure failed with Ti-rich samples.

LA-ICP-MS is able to determine P in solid samples directly without any sample preparation. Thus, Marshall et al. [10] described a new quantification strategy for phosphorus determination in protein by LA-ICP-MS in electrophoresis gel blots. The method developed was applied to the phosphoprotein β -casein with good detection levels [10, 11, 12].

The aim of this work is to develop microanalytical techniques using ICP-MS for the determination of phosphorus concentration in small amounts of protein sample (down to an amount of $1 \mu\text{g}$ or a volume of $1 \mu\text{L}$ tryptic digest). Because the amount of protein sample is limited, special small sample preparation and measurement techniques for the analysis of phosphorus will be investigated. The contamination problem has to be considered in minimizing the analytical techniques with microsample handling. In order to determine the accuracy of the analytical method recovery studies were performed using certified reference material single cell proteins (BCR 273) with a high phosphorus content of $26.8 \pm 0.4 \text{ mg g}^{-1}$. A further reduction of liquid sample volume will be investigated by applying microscale flow injection (μ -FI-ICP-MS). The capability of ICP-SFMS at mass resolution $m/\Delta m \sim 4000$ vs. ICP-QMS at low mass resolution ($m/\Delta m \sim 300$) for phosphorus determination of small protein samples will be studied.

Table 1 Optimized experimental parameters for ICP-SFMS (Element, Finnigan MAT) and ICP-QMS (Elan 6000, PE Sciex) for determination of phosphorus in protein

ICP-MS instrumentation	ICP-SFMS	ICP-QMS
Nebulizer type	Microconcentric	Micromist
Spray chamber	Teflon	Minicyclonic
RF power (W)	1250	1200
Cooling gas flow rate (L min^{-1})	18	14
Auxiliary gas flow rate (L min^{-1})	1.1	1.4
Nebulizer gas flow rate (L min^{-1})	1.32	0.7
Sweep gas flow rate (L min^{-1})	4.82	—
Solution uptake rate (mL min^{-1})	0.05	0.24
Ion extraction lens (V)	2000	66
Mass analyser pressure (mbar)	2×10^{-4}	3.2×10^{-5}
Mass resolution ($m/\Delta m$)	4000	300
Analysis time (min)	5	5
Number of runs	6	20
Number of blocks of runs	5	6

Experimental

Instrumentation

A double-focusing sector-field ICP-MS (ICP-SFMS, Element, Finnigan MAT, Bremen, Germany) at fixed mass resolution ($m/\Delta m \sim 4000$) was used for the measurement of phosphorus concentrations in protein samples. The ICP torch was shielded with a grounded platinum electrode (GuardElectrode, Finnigan MAT). Aqueous solution was introduced into the nebulizer in the flow-injection mode using a peristaltic pump (Perimax 12, Spetec GmbH, Erding, Germany). For comparing measurements the quadrupole-based ICP-MS (ICP-QMS) Elan 6000, Perkin Elmer Sciex, Canada was applied. For the solution introduction of aqueous solution in ICP a microconcentric nebulizer (Micromist) attached to a minicyclonic spray chamber (both from Glass Expansion, Camberwell, Victoria, Australia) and a microconcentric low-flow nebulizer with membrane desolvation (Aridus, CETAC Technologies, Omaha, Nebraska, USA) were used. The experimental parameters of the analytical techniques used are summarized in Table 1.

Standards and reagents

Subboiled nitric acid of suprapure purity from Merck (Darmstadt, Germany) was used for sample digestion. Phosphorus standard stock solution for the calibration procedures was obtained from Merck (Darmstadt, Germany). For all dilutions deionized Milli-Q water ($18 \text{ M}\Omega$) was obtained from a Millipore Milli-Q-Plus water purifier.

Certified reference material (CRM) BCR-273 (single cell proteins with the certified P concentration of $26.8 \pm 0.4 \text{ mg g}^{-1}$) from IRMM (Geel, Belgium) was analyzed by ICP-SFMS and ICP-QMS in order to verify the analytical techniques.

ICP-MS measurement procedure

Optimization of experimental parameters of ICP-MS was performed with respect to the maximal ion intensity of $^{31}\text{P}^+$ using a $1 \mu\text{g L}^{-1}$ phosphorus solution introduced by the Micromist nebulizer. After digestion of phosphorylated peptide and protein samples in a microwave vessel with concentrated nitric acid and dilution the phosphorus concentration was determined using different ICP-MS techniques by external calibration. Microconcentric nebulizer "Aridus" was only attached to ICP-SFMS for determination of phos-

phorus in proteins, when sample quantity was limited, because it provided higher sensitivity (by about two to three times) at lower solution uptake rate (by three times) in comparison to Micromist nebulizer. The combined uncertainty of concentration was calculated according to Ref. [13] taking into account standard deviations of the measured intensity, background and uncertainty of calibration curve.

Samples

The phosphorus concentration was determined in α - and β -casein (from Sigma, Deisenhofen, Germany, with unknown P concentrations) and in a casein mixture from Merck (with a phosphorus content between 0.8 and 0.9%) by ICP-MS. Furthermore, cryoglobulin and phosphorylated proteins were analyzed after protein separation. The 2D-gel electrophoresis of cryoproteins was described by Tissot et al. [14]. Several protein samples were analyzed after separation by 2D-gel electrophoresis and in gel digestion with trypsin (Progenia, Mannheim, Germany). This standard sample preparation procedure was used for protein identification via organic mass spectrometric techniques [15] and for subsequent phosphorus determination via ICP-MS. Phosphorus determination was also performed using the developed analytical methods for 1 μ g of human tau protein (Sigma; from *E. coli*).

Sample preparation

During the preliminary investigation it was revealed that the small quantities of protein samples are inclined to be absorbed on vessel and tube walls, which resulted in non-reproducible sample losses. To digest very small amounts of protein sample down to 1 μ g sample weight 10 μ L concentrated nitric acid was added to the vessel containing the sample and digestion was performed for 10 min at room temperature, and after that the sample was dissolved with 100 μ L Milli-Q water.

Results and discussion

Development of analytical method for phosphorus determination in small amounts of protein sample

The most frequently used ICP mass spectrometers worldwide are quadrupole-based instruments, therefore some

measurements were performed by ICP-QMS. It is well known that the determination of phosphorus in any aqueous solution and also in digested and diluted protein samples by quadrupole-based ICP-MS is quite difficult due to the high background and interference problems at mass 31 u. The background in ICP-QMS depends on the concentration of nitrogen in the samples, i.e. on HNO_3 concentration. A linear calibration curve for phosphorus in the range of 10 to 100 $\mu\text{g L}^{-1}$ was obtained with regression factor of better than 0.998 when using HNO_3 concentration range up to 2%.

Advantageous for the determination of phosphorus is the application of a double-focusing sector field ICP-MS in order to separate disturbing molecular ions from atomic ions of analyte. Figure 1 shows part of a mass spectrum at mass 31 u measured at a mass resolution of $m/\Delta m=4000$ using the ICP-SFMS "Element" from Finnigan MAT when measuring 1 $\mu\text{g L}^{-1}$ phosphorus solution in 1% nitric acid. The P^+ ions are clearly separated from $^{15}\text{N}^{16}\text{O}^+$ and $^{14}\text{N}^{16}\text{OH}^+$ ions, which allows accurate phosphorus determination in protein samples (with only correction of blank resulting from phosphorus impurities in the chemicals used). The detection limit of phosphorus determined in high-purity water using the 3σ criterion (the detection limit is given by $3\sigma_b C_p / I_p$, where σ_b the standard deviation of six independent measurements of the blank value, I_p the measured ion intensities in standard solution with known phosphorus concentration C_p) was 20 ng L^{-1} in ICP-SFMS. In contrast, the detection limit in ICP-QMS was found to be 18 $\mu\text{g L}^{-1}$ (in ICP-SFMS background ion intensities at mass 31 u are: 200 cps vs ICP-QMS: 5000 cps). In protein samples the detection limits are some orders of magnitude higher due to dilution after digestion and higher background intensity. In respect of protein analysis the background equivalent concentrations for phosphorus were determined to be 420 ng g^{-1} and 350 $\mu\text{g g}^{-1}$ for ICP-SFMS and ICP-QMS, respectively.

In order to verify the accuracy of the analytical method developed for small sample sizes a CRM BCR 273 with a

Fig. 1 P determination in protein samples by ICP-SFMS: mass spectrum at mass 31 u, mass resolution: $m/\Delta m=4000$

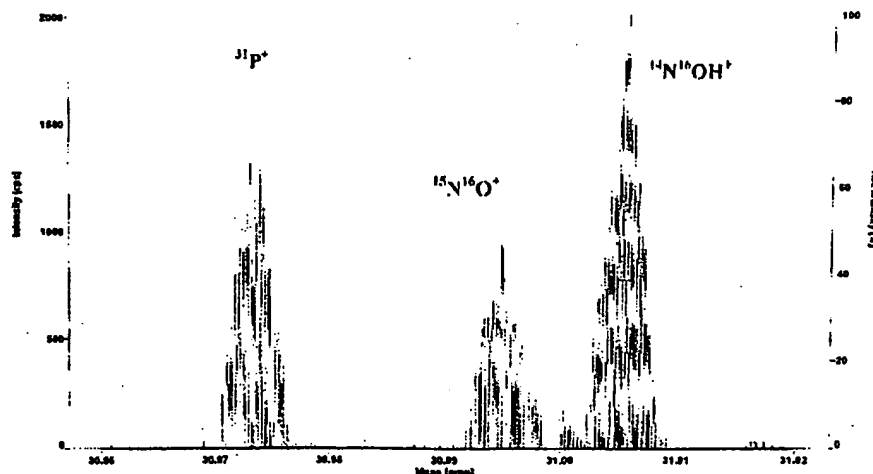


Table 2 Results of phosphorus determination in single cell protein (CRM BCR 273)

	ICP-QMS		ICP-SFMS	
	Measured value ^a (mg g ⁻¹)	RSD (n=5 ^b) (%)	Measured value ^a (mg g ⁻¹)	RSD (n=5 ^b) (%)
40 mg	27.1	2.0	—	—
5 mg	27.8	3.5	24.6	3.0
1 mg	28.6	11.3	27.5	11.1
0.3 mg	30.1	12.1	24.7	9.4

^aCertified value 26.8±0.4 mg g⁻¹^bn is the number of measurements including separate sample digestions

certified P concentration of 26.8±0.4 mg g⁻¹ was analyzed. The recommended sample size of BCR 273 is relatively high at 500 mg due to possible inhomogeneity of sample (and also of phosphorus). In the ideal case, the size of the reference sample used for quality assurance should correspond to the size of the analyzed samples with unknown P concentration. However, significantly smaller samples are available when analyzing the phosphorus concentration in real protein samples. In order to find the minimal sample weight of CRM BCR 273 which still provides reliable results the phosphorus concentration was measured in samples with masses from 40 mg down to 0.3 mg. The results of the determination of P using ICP-QMS and ICP-SFMS as a function of sample size are compared in Table 2. The relative standard deviation (RSD) and measurement accuracy was within 2%, 3.5%, 11% and 12% (five independent measurements including five sample digestions for every sample weight were performed) when analyzing CRM using ICP-QMS for sample sizes of 40 mg, 5 mg, 1 mg and 0.3 mg, respectively. Similar RSD values were observed in ICP-SFMS. Thus, the main component of measurement uncertainty for small samples (1 mg and lower) was caused by sample inhomogeneity, compared with other sources of uncertainty (connected with uncertainty of the calibration curve of up to 2%, standard concentration uncertainty of 0.4% for P, standard deviations of the measured intensity of less than 2%). This result confirms an increasing effect of inhomogeneity of phosphorus in the certified reference material BCR 273 on measurement precision and accuracy with decreasing sample size. An inhomogeneous P distribution in investigated CRM could be demonstrated by measurements using secondary ion mass spectrometry (SIMS).

Application of analytical techniques for phosphorus determination in small amounts of protein sample

Table 3 summarizes the results of phosphorus determination in tau protein, β -casein and a casein mixture from Merck measured mainly by ICP-SFMS. Whereas only 1 μ g was available as the sample weight for different proteins, 1 μ L solution volume of protein digest was investigated

Table 3 Results of phosphorus determination in tau protein, casein, phosphorylated peptides and Mcm3 protein and DNA samples measured by ICP-SFMS

Sample	Molecular weight (Da)	Weight or volume	Phosphorus concentration (g g ⁻¹)
Tau protein	78830	1 μ L	(1.61±0.24)×10 ⁻³
Tau protein digest		1 μ L	(2.32±0.23)×10 ⁻³
β -Casein	25091	1 μ g	(8.3±0.8)×10 ⁻³
β -Casein digest		1 μ L	(7.9±0.8)×10 ⁻³
α -Casein	24513	1 μ g	(6.35±0.4)×10 ⁻³
α -Casein digest		1 μ L	(6.32±0.6)×10 ⁻³
Casein mixture (Merck)		1 μ g	(13.3±1.3)×10 ⁻³
Peptide A ^a		440 μ g	(6.4±0.6)×10 ⁻³
Peptide B ^b		360 μ g	(4.7±0.5)×10 ⁻³
Mcm3 protein		3 μ g (in 30 μ L)	(0.3±0.1)×10 ⁻³
DNA V		100 μ L	(3.51±0.01) μ g mL ⁻¹
DNA IV		100 μ L	(6.07±0.04) μ g mL ⁻¹

^aPeptide A ENDY(PO₃H₂)TNASL
Glu-Asn-Asp-Tyr(PO₃H₂)-Thr-Asn-Ala-Ser-Leu^bPeptide B EPQY(PO₃H₂)EEIPTYL
Glu-Pro-Gln-Tyr(PO₃H₂)-Glu-Glu-Thr-Pro-Thr-Tyr-Leu

using ICP-SFMS. All measured P concentrations are in the mg g⁻¹ range (except the casein mixture with a P content of 1.3%. The P concentration in α -casein at 6.35 mg g⁻¹ is slightly lower and P concentrations in β -casein and the casein mixture at about 8.3 mg g⁻¹ and 13.3 mg g⁻¹ are slightly higher than expected from comparison with previous results [3]. Thus, Bandura et al. [3] measured phosphorus content of 4.94 mol mol⁻¹ and 8.8 mol mol⁻¹ for regular β -casein and α -casein, respectively (resulting in 6.1 mg g⁻¹ for β -casein and 11.1 mg g⁻¹ for α -casein with account to protein masses given in Table 3). The results deviation could be due to the fact that different genetic modifications of caseins have different phosphorylation degree, and the material analyzed in the present work differed from those studied in Ref. [3]. A lower phosphorus concentration was measured in tau protein, probably because of a low extent of phosphorylation, although multiple phosphorylation sites have been identified for this protein [15].

Furthermore, small sample volume of tryptic digests of brain protein samples were analyzed. Table 4 presents the

Table 4 Results of phosphorus determination in protein samples (Cryoglobulins, 1 μ L) after 2D gel electrophoresis and trypsin digestion by ICP-SFMS

Sample	Phosphorus weight (g)	Phosphorus concentration (g g ⁻¹)
1	(1.2±0.3)×10 ⁻¹⁰	(6.0±1.5)×10 ⁻³
2	(1.82±0.27)×10 ⁻¹⁰	(9.1±1.3)×10 ⁻³
3	(1.59±0.24)×10 ⁻¹⁰	(8.0±1.2)×10 ⁻³
4	(1.95±0.30)×10 ⁻¹⁰	(9.8±1.5)×10 ⁻³
5	(2.07±0.31)×10 ⁻¹⁰	(10.4±1.5)×10 ⁻³

results of phosphorus determination in phosphorylated peptides, protein Mcm3 and two DNA samples. The sample weight varied between 3 μg and 440 μg . The phosphorus concentrations in two phosphorylated peptides were determined to be 0.64% and 0.47%, respectively. In the cell cycle regulation protein Mcm3, a phosphorus content about one order of magnitude lower was found.

Application of flow injection for determination of phosphorus in proteins

A further reduction of sample amount is possible by applying microscale flow injection ($\mu\text{-FI-ICP-MS}$) as described in Ref. [8]. For these measurements of transient ion signals a sample loop of 20 μL using an HPLC injection valve was applied. Transient signals of a $10\text{ }\mu\text{g L}^{-1}$, $20\text{ }\mu\text{g L}^{-1}$, and $50\text{ }\mu\text{g L}^{-1}$ P solution (sample loop: 20 μL , sample size: 200 pg, 400 pg and 500 pg, respectively) were measured by ICP-SFMS. The transient signals of phosphorus in protein solutions were measured with a precision (RSD of six replicates) of 3.9%, 3.2% and 2.6%, respectively. In comparison, a precision of 1.98% was observed for diluted CRM BCR 273 sample with a phosphorus concentration of $610\text{ }\mu\text{g L}^{-1}$.

Figure 2 presents the flow injection diagram measured by ICP-SFMS obtained when measuring protein samples without digestion (sample 1) and after digestion (samples 18 and 24). Only a single measurement with a sample loop of 20 μL was performed because of limited sample size. Sample 3 was diluted with 10% nitric acid, samples 4 and 5 were digested with concentrated nitric acid for 10 min and then diluted with Milli-Q water. In the case of undigested sample (1) the phosphorus ion intensity increased slowly due to protein absorption on the tube walls. In addition, casual peaks of phosphorus signal were observed after the sample was consumed (during the washing time) due to the washout of absorbed proteins. These undesirable effects were significantly reduced when samples were digested thus allowing reproducible results.

We attempted to measure phosphorus in five different cryoglobulin samples separated by 2D gel electrophoresis

and digested with trypsin. Due to a very low solution volume of 1 μL only the ICP-SFMS measurements were performed by flow injection whereby the relative standard deviation between 14 and 25% is relatively high. The determined phosphorus weight in different samples was very similar and varied between $1.2 \times 10^{-10}\text{ g}$ and $2.07 \times 10^{-10}\text{ g}$. The measured phosphorus concentration in blank gel (without any protein spots) used for 2D gel electrophoresis was 0.11 mg g^{-1} , which corresponded to the phosphorus measured in cryoglobulin samples with account to dilution factor during the sample preparation procedure (about 1 μg of gel from a spot containing cryoglobulin was used for analysis, resulting in about $1.1 \times 10^{-10}\text{ g}$ of phosphorus, which originated from the gel). The determination of phosphorus in small protein samples like cryoglobulins is extremely difficult due to the high P concentration in cerebrospinal fluid, in the gel used and in trypsin. The analysis in such a small volume and also considering the possible contamination during sample preparation requires further careful investigations.

Conclusions

ICP-MS is a powerful trace analytical method for the determination of phosphorus in small amounts of biological samples. By application of microanalytical techniques with ICP-MS using micronebulization it is possible to perform multielement analysis on protein samples down to 1 μg . The most important problems are the inhomogeneity of standard reference materials and biological samples and possible contamination during sample preparation. It was demonstrated that sector field ICP-MS, and with certain reservations ICP-QMS also, yielded adequate results for small sample sizes. In general, ICP-SFMS with micronebulization can be recommended as "analytical protocol of choice" for phosphorus analysis in small protein samples. In the future work, performance of ICP-QMS with collision cell will be studied for application in phosphorus analysis.

References

1. Sefton BM, Hunter T (1998) (eds) Protein phosphorylation. Academic Press, San Diego, CA
2. Davies MJ, Dean RT, Davies D (1998) Radical-mediated protein oxidation: from chemistry to medicine. Oxford University Press, Oxford, UK
3. Bandura DR, Baranov VI, Tanner SD (2002) Anal Chem 74:1497-1502
4. Baranov VI, Quinn ZA, Bandura DR, Tanner SD (2002) J Anal At Spectrom 17:1148
5. Wind M, Edler M, Jakubowski N, Lindscheid M, Wesch H, Lehmann WD (2001) Anal Chem 73:29
6. Wind M, Wesch H, Lehmann WD (2001) Anal Chem 73:3006-3010
7. Broekaert JAC (2002) Analytical atomic spectrometry with flames and plasmas. Wiley-VCH, Weinheim
8. Becker JS, Dietze H-J (2000) Int J Mass Spectrom 195/196:1
9. Hill SJ (1999) (ed) Inductively coupled plasma spectrometry and its applications. Sheffield Academic Press, Sheffield, UK

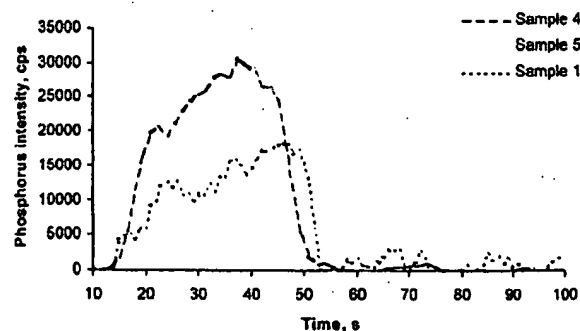


Fig. 2 Transient signal of phosphorus on protein (cryoglobulin) solutions measured by ICP-SFMS using flow injection (single measurement, sample loop 20 μL , P concentration 17 mg L^{-1} – 33 mg L^{-1})

10. Marshall P, Heudi O, Bains S, Freeman HN, Abov-Shakra F, Reardon K (2002) *Analyst* 127:459
11. Boulyga SF, Pickhardt C, Becker JS, Buddrus S, Przybylski M, Becker JS (2002) 8th Int Conf on Plasma Source Mass Spectrometry, September 8–13, 2002, Durham, UK, Book of abstracts, p 49
12. Bandura DR, Baranov VI, Omatsky OI, Quinn ZA, Tanner SD (2002) 8th Int Conf on Plasma Source Mass Spectrometry, September 8–13, 2002, Durham, UK, Book of abstracts, p 50
13. Taylor RN, Warneke T, Milton JA, Croudace IW, Warwick PE, Nesbitt RW (2001) *J Anal At Spectrom* 16:279
14. Tissot J-D, Invernizzi F, Schifferi JA, Sperteni F, Schneider P (1999) *Electrophoresis* 20:606
15. Rossier JS, Youhnovski N, Lion N, Damoc E, Becker S, Raymond F, Girault HH, Przybylski M (2003) *Angew Chem Int Ed Engl* 115:55–60